Genetic inhibition of telomerase results in sensitization and recovery of breast tumor cells

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Abstract

Telomerase, a ribonucleoprotein enzyme minimally composed of an RNA template (human telomerase RNA) and a catalytically active protein subunit (human telomerase reverse transcriptase), synthesizes telomeric repeats onto chromosome ends and is obligatory for continuous tumor cell proliferation. Telomerase is an attractive anticancer therapeutic target because its activity is present in >90% of human cancers, including >95% of breast carcinomas. Traditional chemotherapies lack the ability to effectively control and cure breast cancer, in part because residual cells are often resistant to DNA-damaging modalities. Although numerous telomerase inhibition strategies cause cancer cells to undergo apoptosis or senescence, there is often a lag period between the beginning of the treatment regimen and a biological effect. Thus, our goal for these studies was to show that effectively blocking telomerase genetically together with standard chemotherapeutic agents, doxorubicin/Adriamycin or Taxol, would increase the sensitization and efficacy for triggering senescence and/or apoptosis in cultures of breast cancer cells while reducing toxicity. We find that blocking telomerase in breast tumor cells substantially increases the sensitization at lower doses of Adriamycin or Taxol and that the kinetics of senescence/apoptosis is more rapid at higher concentrations. Combined with telomerase inhibition, Taxol treatment induced both apoptosis (its typical cell fate) and senescence, both at high enough levels to suggest that these two cellular responses are not mutually exclusive. Genetic inhibition of telomerase is eventually reversed due to up-regulation of endogenous telomerase activity without a net change in telomere length, suggesting that telomerase inhibition itself, not necessarily short telomeres, is important for sensitization. [Mol Cancer Ther 2009;8(5):1319–27]

Introduction

The primary treatment for breast cancer consists of surgery and adjuvant therapies, including chemotherapy, hormone therapy, and localized radiation, many of which often result in high toxicity and nonspecific side effects. Therefore, despite the initial success of these clinical approaches, the frequent recurrence of breast cancer indicates that resistance to therapy is common and is one of the main causes of cancer treatment failure. Metastatic disease is one of the major challenges for breast cancer treatment today (1), essentially as a result of evasion of traditional chemotherapy and acquired drug resistance.

Cancer is the second leading cause of death, and the occurrence of cancer is strongly tied to advanced age (2). Genomic instability, including telomere dysfunction, is thought to be a major contributory factor in aging and progression to cancer. Telomeres are the natural ends or caps of linear chromosomes that are composed of both repeated DNA elements and specific DNA-binding proteins (3). Although size and sequence vary among species, human telomeres are composed of ∼10 to 15 kb of tandem hexameric repeats, 5′-TTAGGG-3′ (4). Telomeres serve to maintain and protect the ends of chromosomes from end-to-end fusions (5) and from being recognized as double strand breaks, thereby preventing the nonhomologous end joining that result in telomere fusions (6).

Telomerase, a ribonucleoprotein enzyme minimally composed of an RNA template [human telomerase RNA (hTR)] and a catalytically active protein subunit [human telomerase reverse transcriptase (hTERT); ref. 7], synthesizes telomeric repeats onto chromosome ends and is obligatory for continuous tumor cell proliferation as well as malignant progression of breast cancer cells. Telomerase is an attractive anticancer therapeutic target because its activity is present in >90% of human cancers, including >95% of breast carcinomas, but it is undetectable in most somatic cells. As such a ubiquitous marker of a majority of cancers, therapeutic opportunities would be greatly improved if a synergism between telomerase inhibition and established antitumor strategies could be shown. The rate-limiting component of the telomerase complex is the hTERT protein; therefore, the expression of the hTERT gene within the cells is critical for telomerase activation.
Many tools have been used to target telomerase RNA and hTERT, such as oligonucleotides, ribozymes, or small interfering RNA, which have been effective in knocking down expression without reaching 100% efficiency (8). Similarly, previous reports have shown that dominant-negative hTERT (DN-hTERT) is able to induce telomerase inhibition, telomere shortening, and apoptosis of tumor cells (9, 10), whereas other studies have reported the induction of cellular senescence depending on the cell line used (11). It is still unclear what makes a cell preferentially undergo senescence versus apoptosis in response to telomerase inhibition, although there is a role for telomere dysfunction and p53 (11, 12).

Previous studies fail to thoroughly provide the cellular consequences of using telomerase inhibition to sensitize cells while using only a very small range of drug concentrations. To comprehensively examine the biological effects of telomerase inhibition in breast tumor cells, telomere maintenance was blocked by using DN-hTERT expression in breast tumor cells. Here, we find that telomerase inhibition, even at less than complete knockout, induces telomere shortening and slowed growth, and when used in combination with doxorubicin/Adriamycin or Taxol, we observe high levels of senescence or apoptosis, respectively. Unexpectedly, we find the reversion of telomerase inhibition over time, indicating that the mechanism of telomerase up-regulation in the presence of DN-hTERT involves an increase in endogenous hTERT mRNA expression. Collectively, our data suggest that when telomerase inhibition is used in an adjuvant setting, with diverse acting chemotherapeutic agents, there is potent chemotherapeutic sensitization characterized in part by widespread senescence and/or apoptosis.

Materials and Methods

Cell Culture

The MCF-7 human breast adenocarcinoma cell line was cultured in RPMI 1640 containing 5% FBS and 800 ng/mL gentamicin and maintained at 37°C with 5% CO2. Cells were counted using a hemocytometer and population doublings were calculated as before (13).

Retroviral Infections

The pBABEpuro, GFP-hTERT, or DN-hTERT vectors were transfected into the 293T competent packaging cell lines along with two additional vectors encoding critical packaging proteins (pC1 and VSVG) using Fugene 6 Transfection Reagent (F. Hoffman-La Roche; ref. 14). Retroviral supernatants were collected at 24, 48, and 72 h after transfection; filtered with a 0.45-μm filter; and added to MCF-7 cultures

Telomeric Repeat Amplification Protocol

Using the TRAP-eze telomerase detection kit (Millipore), telomerase activity was determined according to the manufacturer’s protocol as before (12). Approximately 100,000 cells were lysed and subjected to the telomeric repeat amplification protocol assay using a γ-32P-ATP TS primer for telomerase-mediated extension and PCR amplification. PCR products were run on a 10% polyacrylamide gel. Telomerase activity levels were quantitated using ImageQuant software (Molecular Dynamics), which calculates the ratio of the internal control (36 bp) to the laddered telomerase products. The telomerase activity for the DN-hTERT clones was assessed and presented as a percentage of that measured in the pBABE empty vector controls.

Reverse Transcription-PCR Analysis of hTERT

RNA isolation was done using the RNeasy Mini kit with QiAshredders (Qiagen) according to the manufacturer’s protocol. Using the RETROscript reverse transcription-PCR (RT-PCR) kit (Ambion), 1 to 2 μg of total RNA were reverse transcribed using random decamers. PCR amplification was accomplished using traditional techniques as described previously (12), with amplification of 18S as a control, or by using real-time RT-PCR. Primers used for the genes of interest were as follows: for endogenous hTERT amplification, hTERT (5′-GACTCGACACCGTGTTGACCC-3′), Endo#1 (5′-ACCTAGGCGCCGGTGACAG-3′). For exogenous hTERT amplification primer hTERT coupled with pBabe (5′-GACACACATTCCACAGGTG-3′). For hTR amplification, R3C (5′-GTGTTGCTCTAGATGAAGCCGTTGAA-AG-3′), F3B (5′-TCTAACCCTAAGCGAAAGGCGTATG-3′). For gel-based RT-PCR, reaction products were resolved on a 2.5% agarose gel in the presence of ethidium bromide and visualized under UV light on an imaging system (Alpha Innotech Corporation). For real-time RT-PCR, both a standard curve for optimized cDNA amplification and a primer disassociation were generated. Relative quantitative PCR was then carried out using an Applied Bioscience 7900HT machine using the Express SYBR GreenER qPCR Supermix (Invitrogen). The resulting data were analyzed using the 2−ΔΔCt method where the endogenous ribosomal 18S (Ambion) gene expression was used as a standard and the pBABE vector control for normalization purposes.

Western Analysis

Parental MCF-7 cells along with isogenic derivatives were lysed for 30 min on ice in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1% aprotinin, and 100 mmol/L DTT]. Ten to 50 μg of total cell lysate were resolved by SDS-PAGE, electrotransferred onto nitrocellulose, and probed with the following primary antibodies: p53 (BD Biosciences; 1:5,000 dilution); p21 (BD Biosciences; 1:5,000); β-actin (Sigma; 1:5,000); Hsp90, Hsp70, p23 (David O. Toft, Mayo Clinic; 1:5,000); and/or hTERT (Rockland; 1:1,000), followed by either antimouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase (Bio-Rad). Complexes were detected with the Supersignal West Pico Chemiluminescent kit (Pierce Bio-technology) and exposed to autoradiographic, Kodak film.

Senescence-Associated β-Galactosidase

Cells were washed twice in PBS, fixed for 3 to 5 min at room temperature with 2% formaldehyde/0.2% glutaraldehyde in
PBS, and washed twice with PBS. The cells were then incubated for up to 16 h at 37°C with freshly prepared β-galactosidase substrate (12, 15). Following two PBS washes, representative images were captured using an OLYMPUS 1× 70 fluorescent microscope.

**Telomere Amount and Length Assay**

Measurement of telomere lengths was conducted using telomere amount and length assay, an in-solution hybridization protocol (16). Briefly, up to 5 μg of genomic DNA was digested using a cocktail of HaeIII, HhaI, and Hinfl restriction enzymes for 4 h at 37°C. Digested genomic DNA was combined with 1.5 ng of 32P-labeled telomere-specific probe (TTAGGG)4, denatured at 98°C for 6 min, hybridized for 18 h at 55°C, and cooled to 4°C for at least 5 min. Samples were resolved on a 0.8% agarose gel for 18 h at 60 V, dried for 1.5 h, and exposed to a phosphorimager screen (Molecular Dynamics). The resulting image was analyzed by the ImageQuant software (Molecular Dynamics), and calculations of telomere length were done as before (16).

**Terminal Deoxyribonucleotide Transferase–Mediated Nick-End Labeling Assay**

MCF-7 cytospin preparations of both floating and adherent cells were fixed in 4% formaldehyde and washed twice in PBS. The cells were then postfixed with an acetic acid/ethanol (2:1) solution for 5 min at −20°C and again washed with PBS. The subsequent steps were done in a covered humidified chamber at room temperature, which included blocking in 1 mg/mL bovine serum albumin for 30 min and incubating with terminal transferase (F. Hoffman-La Roche) and fluorescein 12-dUTP (F. Hoffman-La Roche) for 60 min at 37°C in the dark. Cells were then washed in PBS and mounted in Vectashield (Vector Labs), and representative images were captured using an OLYMPUS 1× 70 fluorescent microscope.

**Adriamycin Treatment and Colony-Forming Efficiency Assay**

To compare breast cancer cell growth after acute treatment with Adriamycin, 2,000 cells were plated in 10-cm² dishes. The next day, cultures were treated with a range of concentrations of Adriamycin for 2 h. Six different MCF-7-derived cell lines were tested [MCF-7 parental, MCF-7 DN-hTERT clones (DN2, DN6, DN9), and vector-only controls (pBABE2, pBABE3)] with each drug concentration tested in triplicate for each line. The cells were maintained for 10 d followed by a 10-min fixation in 3.7% formaldehyde and crystal violet staining. Colonies were counted using the mammalian cell counter ColCount (Discovery Technology International). A two-tailed t test was done to assess statistical significance.

**Results**

**DN-hTERT Inhibits Telomerase Activity and Induces Telomere Shortening**

To further characterize the consequence of telomerase inhibition in breast cancer cells using DN-hTERT, we used the catalytically inactive DN-hTERT (9) with two substitutions in the third reverse transcriptase motif of hTERT. At positions 712 and 713, the aspartic acid and valine residues were changed to alanine and isoleucine, respectively. Telomerase activity was assessed using 250, 500, and 1,000 cell equivalents. The percent knockdown (%KD) of activity in relation to the vector control are shown below each group of three lanes. The percent knockdown (%KD) of total protein were subjected to Western analysis for hTERT protein levels. Immunoblots were probed with antibodies for hTERT and for β-actin, which served as a loading control. Expression of a GFP-hTERT (wild-type) was accomplished in MCF-7 cells either with or without an untagged DN-hTERT, showing a change in fluorescence pattern after DN-hTERT expression, from nuclear (left) to cytoplasmic (middle). Phase contrast images is shown for nuclear/cytoplasmic delineation (right).
switched to alanine and isoleucine, respectively (Fig. 1A). The DN-hTERT and empty vector controls were retrovirally infected into MCF-7 cells, and telomerase activity was analyzed in clonal isolates. Introduction of DN-hTERT resulted in a repeatable and significant reduction in telomerase activity levels in all of the MCF-7 clonal populations tested, although with some variability between clonal populations. The greatest knockdown of activity was found in clones 2 (12% of control), 6 (27%), and 10 (26%) noted as DN2, DN6, DN10; Fig. 1B), whereas two pBABE clones (pBABE2 and pBABE3) showed no observable decline in activity relative to parental baseline controls. The growth of DN-hTERT clones exhibited a decline over time compared with pBABE controls (not shown), which correlated with telomerase activity levels.

Assessment of the hTERT RNA levels for each clonal population shows that the DN-hTERT was expressed in each clone without altering endogenous hTERT expression (data not shown). Western analysis revealed a decrease in overall hTERT levels in all clones expressing DN-hTERT (Fig. 1C) compared with the vector-only control, suggesting that DN-hTERT itself is unstable and its expression results in a decline in endogenous hTERT protein levels. As predicted, these differences correlated with the telomerase activity levels observed, suggesting that those clones with greater expression of DN-hTERT also had lowest levels of telomerase activity. To determine if the DN-hTERT was able to affect wild-type forms of hTERT localization in MCF-7 cells, we established a stable MCF-7/GFP-hTERT–expressing cell line, where all of the GFP-hTERT was expressed in the nucleus (Fig. 1D, left). When an untagged DN-hTERT was co-expressed in the MCF-7/GFP-hTERT cells, we found that the GFP signal was predominantly cytoplasmic (Fig. 1D, middle), suggesting a possible mechanism for how DN-hTERT affects wild-type forms of hTERT. The DN-hTERT was able to induce the translocation of the GFP-hTERT from the nucleus to the cytoplasm.

As predicted, telomere lengths of MCF-7 cells expressing DN-hTERT decreased over time (Fig. 2A). Compared with parental MCF-7 and MCF-7 ectopically expressing hTERT or pBABE control vectors, the DN-hTERT clones displayed a marked decrease in telomere length of 1 kb or more, with the DN2 clone being the most significantly shortened. The amount of shortening seems to correlate with level of DN-hTERT expression (Fig. 1C). Interestingly, the relatively short length of the telomeres does not completely inhibit growth as clones continue to grow with telomeres as small as 2.4 kb (growth data not shown).

**DN-hTERT Causes Sensitization of Breast Tumor Cells to Adriamycin**

Previous studies have established increased sensitivity to chemotherapeutic drugs after telomerase inhibition in a variety of tumor cell lines (17–20). Therefore, we wanted to determine if expression of DN-hTERT causes increased sensitization in breast cancer cells, specifically MCF-7 cells. We examined the ability of MCF-7 cells to survive and proliferate using colony-forming assays after treatment with the DNA-damaging agent Adriamycin (doxorubicin), an anthracycline antibiotic and topoisomerase II inhibitor. Three of the DN-hTERT clones with differing amounts of telomerase activity and telomere lengths were tested about 2 months postinfection. In comparison with the MCF-7 and pBABE cell lines, all of the DN-hTERT clones exhibited a greater sensitivity to Adriamycin as seen by the considerable reduction in the average number of colonies (Fig. 2B).

Clonal growth rates were significantly lower than controls for all Adriamycin concentrations tested except 0.0625 μmol/L in the DN9 clone, which has longer telomeres and less DN-hTERT expression than the other clones (hence, only slightly inhibited telomerase activity), yet still showed sensitization as low as 0.125 μmol/L Adriamycin. This increased sensitivity to Adriamycin implies an increased susceptibility of shortened telomeres to Adriamycin and thereby DNA damage.

**DN-hTERT Clones Recover by Telomerase Reactivation**

Instead of the projected lag time of growth and telomere shortening followed by death or senescence, long-term culture of the DN-hTERT lines resulted in the emergence of surviving cells that reverted back to normal phenotype and growth rate. We observed a “crisis” period, where cell death and division were essentially equal. Furthermore,
these cells regained higher levels of telomerase activity than those found in normal MCF-7 and pBABE control cell lines (Fig. 3A). Although activity levels exceeded the vector controls, all of the clones seemed to have undergone only a slight lengthening in overall telomere length compared with prerecovered cells (e.g., referred to as “crisis” cells; Fig. 3B), suggesting that the increase in telomerase may be required for other functions in addition to telomere maintenance. Recent studies have shown that telomerase will elongate only those telomeres that are most likely involved in the chromosomal abnormalities that cause genomic instability (21, 22). Thus, although the overall length of the telomeres was not significantly elongated compared with telomerase-inhibited clones (i.e., during crisis), telomerase may preferentially elongate the shortest telomeres within the cell.

Mechanism of Telomerase Recovery

Previous studies have reported a reactivation of telomerase in human leukemia cell lines (acute myelogenous leukemia and promyelocytic leukemia) and a murine kidney tumor cell line after telomerase inhibition with a dominant-negative TERT (20, 23–25). To uncover the basis for this recovery, we examined the RNA levels of endogenous and exogenous hTERT using real-time RT-PCR (Fig. 3C). We wanted to compare expression levels before and after recovery, as well as detect differences in RNA levels between the clones. Interestingly, we found no dramatic up-regulation in endogenous levels of hTERT mRNA compared with pBABE vector controls, whereas exogenous DN-hTERT remains overexpressed (Fig. 3C). One possible mechanism for elevated telomerase activity could be up-regulation of the RNA component of telomerase, hTR, endogenously. Again, using real-time RT-PCR, we found no substantial alteration in human telomerase RNA expression levels in any of the DN-hTERT clones when compared with the vector-only controls (Fig. 3C).

The levels of hTERT protein were assessed in five of the DN-hTERT clones and we found similar levels of hTERT as the pBABE clone 3 (Fig. 3C), which translates into considerably higher levels of hTERT compared with those seen previously (see Fig. 1). Thus, even in the presence of DN-hTERT, endogenous hTERT protein is capable of being up-regulated to compensate for the degradation imposed by DN-hTERT. To define the mechanism for elevated endogenous hTERT stability and function, we hypothesized that because the Hsp90 chaperone complex is responsible for telomerase assembly, these chaperones may also stabilize hTERT and protect endogenous telomerase from DN-hTERT-induced degradation. We assessed the protein levels of Hsp90, Hsp70, and the Hsp90 cochaperone p23 as members of...
the telomerase assembly pathway, essentially showing no change in chaperone levels in any of the recovered DN clones (data not shown), suggesting that these chaperones are not affected by the increased stability of telomerase in recovered clones.

**Sensitization of DN-hTERT-Expressing Cells during Crisis and Postrecovery**

We tested if DN-hTERT expression resulted in sensitization of MCF-7 clonal populations to Adriamycin and Taxol during crisis and after reactivation of telomerase postrecovery. Crisis was defined as those cells that showed DN-hTERT effect (i.e., no up-regulation of endogenous hTERT). Cells were exposed to acute treatment with various concentrations of Adriamycin (0.0–0.75 μmol/L) or Taxol (0.0–0.15 μmol/L), and then at days 2 and 4, growth and frequency of senescence and apoptosis were assessed. We tested four of the clones but show results for only the DN2 clone, which not only had the highest degree of telomerase inhibition but also the most significant sensitization. Compared with the pBABE control cells, only the DN2 clones in crisis (i.e., with inhibited telomerase activity) showed sensitization to both Adriamycin and Taxol in terms of growth for both days and every concentration tested, whereas there is essentially no effect on the growth of the recovered cells (Fig. 4).

The DN2 cells in crisis displayed significantly higher levels of senescence for every concentration of drug administered at both 2 and 4 days posttreatment (Fig. 5). The kinetics of senescence seems to be much more rapid than the vector controls, although the basal level of senescence for the DN2 clone is nearly 20%. For example, at the clinically relevant dose of Adriamycin (0.75 μmol/L), nearly 90% of the cells show senescence-associated β-galactosidase (SA-β-gal) staining (i.e., senescence) at 2 days, whereas the Adriamycin-treated pBABE clone shows <40% senescence. In addition, there seems to be significant sensitization to Adriamycin in Taxol in DN-hTERT-expressing cells. For example, treatment of the DN2 clone with Adriamycin at 0.08 μmol/L or Taxol at 0.015 μmol/L causes about 40% senescence compared with <5% for the drug-treated pBABE vector control (Fig. 5). Although the mode of action of Taxol on MCF-7 cells is typically apoptosis, we observe a relatively high level of senescence after treatment of the DN-hTERT-expressing cell line. The telomerase-recovered cells show essentially no statistically significant differences in the frequency of induction of senescence.

We also examined the induction of apoptosis after treatment with Adriamycin or Taxol. Importantly, both floating and adherent cells were collected by cytospin for analysis of apoptosis. Neither pBABE control or the DN2 crisis cells revealed considerably high levels of cell death at day 2 or day 4 after treatment of Adriamycin as expected due to wild-type p53 function (12), but Taxol treatment produced significantly higher levels of apoptosis in the DN2 crisis cells at concentrations as low as 0.015 μmol/L (Fig. 6). As in the case for growth and senescence, there seems to be no significant difference between vector controls and recovered DN clones for either Adriamycin or Taxol treatments.

**Discussion**

Telomerase is expressed in nearly 90% of human tumors and up-regulated in 95% of breast carcinomas, making it extremely promising for aiding in cancer diagnosis and prognosis, as well as a likely target for cancer treatment. Other studies have shown that telomerase activity can be successfully targeted and knocked down in combination with various chemotherapeutics in several different cell lines. Unlike our study, these reports were limited to only one or two drug concentrations without reporting the cellular consequence of treatment, sensitization, recovery, or resistance (20, 23–25). The data presented here are unique in that a lower range of drug concentrations was shown to sensitize cells with accelerated kinetics compared with control cells, suggesting that breast tumor cells are more sensitive to the doxorubicin/Adriamycin DNA-damaging agent and the microtubule poison Taxol after telomerase inhibition.

To address the mechanism of dominant-negative telomerase, we show that DN-hTERT is able to cause the cytoplasmic relocation of wild-type hTERT in MCF-7 cells. Coupled with the reduction of total hTERT protein in DN-hTERT-expressing MCF-7 cells and our data indicating that
DN-hTERT induces cytoplasmic degradation of wild-type telomerase, it is clear that one of the predominant mechanisms for DN-hTERT function is to reduce endogenous levels in a ubiquitin-mediated manner. Those DN clones that recover telomerase function seem to have evaded this degradation process without elevating endogenous hTERT mRNA levels, causing a significant increase in overall telomerase activity levels. Interestingly, there seems to be no increase in telomere lengths with 15- to 20-fold elevated telomerase activity.

Here, we find that DN-hTERT causes a decrease in telomerase activity, telomere shortening, and sensitization to mainstay breast cancer chemotherapeutic compounds, which is then followed by clonal resistance to both the dominant-negative effect and sensitization. For clones DN2 and DN6, both of which had the highest levels of DN-hTERT expression, significant sensitization was observed at Adriamycin dosages as low as 0.08 μmol/L and at Taxol doses at 0.015 μmol/L, whereas others with intermediate telomerase knockdown showed only modest sensitization. This shows that although complete knockdown of telomerase function is not required to effectively cause telomere shortening, we find that the more inhibited telomerase is, the more sensitive the cells are to Adriamycin and Taxol. One report specifically suggests that the DN-hTERT-induced sensitization effect was due to the resultant shortened telomeres (20), whereas our data argue against that for at least two reasons. First, the recovered populations with high telomerase activity and minimally elongated telomeres (i.e., telomere lengths similar to telomerase inhibited clones) lack sensitivity to Adriamycin or Taxol. Second, all DN-hTERT clonal populations had similar telomere lengths and only differed in the level of telomerase knockdown, suggesting that activity levels rather than global telomere lengths are more important for sensitization to Adriamycin and Taxol. Our results also dramatically differ from another study that found HeLa cells sensitive to only Adriamycin and not to Taxol (18), implying some cell-type specificity for Taxol.

![Figure 5](image1.png)  
**Figure 5.** Increased senescence after treatment with Adriamycin and Taxol in DN-hTERT cells during crisis but not after recovery. DN-hTERT crisis or recovered cells treated with either Adriamycin (A) or Taxol (B) were tested for senescence using SA-β-gal staining. Senescence was calculated by counting at least three independent fields of 100 cells to determine SA-β-gal-positive cells. *, significance of $P < 0.05$.

![Figure 6](image2.png)  
**Figure 6.** Taxol and DN-hTERT together induce increased levels of apoptosis in crisis cells but not after recovery. DN-hTERT crisis or recovered cells acutely treated with either Adriamycin (A) or Taxol (B) were tested for apoptosis using TUNEL staining, and both floating and adherent cells were collected by cytospin. Apoptosis was calculated by counting at least three independent fields of 100 cells to determine the %TUNEL positive (i.e., apoptotic). *, significance of $P < 0.05$. 

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treatment with breast cancer cells being more sensitive to Taxol in general.

Although cells treated with Taxol typically undergo apoptosis, we observe high levels of senescence for both Adriamycin- and Taxol-treated cells expressing DN-hTERT. Interestingly, Taxol-treated DN-hTERT cells also undergo apoptosis, suggesting that senescence and apoptosis are not mutually exclusive in breast tumor cells under these conditions. Comparing the numbers, we find that DN-hTERT cells treated with Taxol have 60% to 80% senescence (SA-β-gal positive) and 50% to 60% apoptosis [terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) positive], suggesting that the two cellular responses may be able to occur simultaneously after exposure to Taxol. This was an unexpected result, given that cell death and drug-induced senescence/stasis have been thought to be mutually exclusive cellular responses to chemotherapy. As expected for Adriamycin-treated cells, it seems that cell fate is either senescence or apoptosis based on the quantification, consistent with the previous dogma in the literature (26, 27). This is a potentially intriguing result, which we are currently exploring.

Approximately 4 months postinfection of the DN-hTERT transgene, all of the clones recovered telomerase activity, although telomere length was only modestly elongated. The recovery of cells after expression of a catalytically inactive form of hTERT was used to block telomerase has been reported, and these groups propose their own theory on the mechanism, from activation of endogenous TERT (25, 28) to selection of cells with silenced DN-TERT transgene (29). Whereas the molecular basis of this recovery is clearly not well understood, our data suggest that it is not due to the loss of the transgene but to up-regulation of the endogenous hTERT protein and increased stability of the hTERT protein in the absence of significant telomere elongation. Interestingly, the elevated telomerase expression was not observed for any of the clones at the transcriptional level. The mechanism for the ability of DN-hTERT to down-regulate endogenous telomerase has been partially explained by our experiments shown here (see Fig. 1D) and recent research in our laboratory that indicates the DN-hTERT induces the cytoplasmic shuttling and degradation of wild-type hTERT. It is formally possible that in response to the degradation of endogenous hTERT, specific DNA damage signals as a result of telomere attrition are able to induce the stabilization of the hTERT protein or an increase in chaperone-mediated telomerase assembly. We speculate that this up-regulation may in part be due to functional wild-type p53, which may be capable of directly or indirectly inducing a specific transcription factor responsible for hTERT transactivation. In addition, it seems that the recovered DN-hTERT cells are capable of evading the degradation of endogenous wild-type hTERT through mechanisms that are as yet unexplored.

Given that we observe no significant telomere elongation even when telomerase is up-regulated 20-fold (see Fig. 3A and B), it seems that the resistance to chemotherapy is unrelated to overall telomere length. In fact, it may be that up-regulation of telomerase itself, independent of its role in telomere maintenance, is capable of providing an alternative pathway to resistance that does not require elongating telomere lengths. There is evidence that telomerase can function in a number of alternative signaling mechanisms, some of which could be involved in chemotherapeutic resistance, including DNA damage response and DNA repair (30).

In conclusion, the combination of DN-hTERT with the standard chemotherapies Adriamycin and Taxol is highly effective at sensitizing breast tumor cells to apoptosis and/or senescence. It is important to note that the genetic inhibition of telomerase offers only a relatively small window of opportunity, given the occurrence of therapeutic resistance. However, because of this lag period between DN-hTERT–mediated inhibition and eventual resistance, the clinical relevance of genetic inhibition of telomerase may be that complete telomere erosion would not be necessary, and patients could receive combinatorial therapy after only a modest telomere shortening has occurred. Thus, it may be that the combination of telomerase inhibition and lower doses of mainstay breast cancer drugs would reduce the side effects and toxicity observed with just a single dose of chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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